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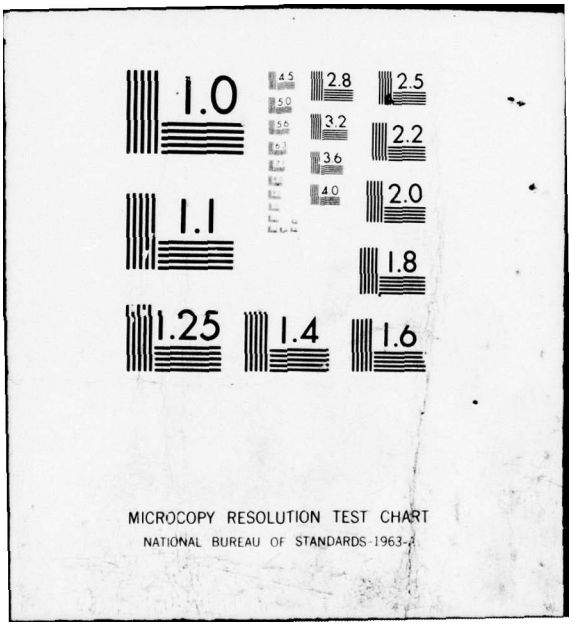
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STRUCTURE AND FUNCTION OF COBROTOXIN

by

Chen-chung Yang, D. Med. Sc.

Professor of Biochemistry
Institute of Molecular Biology
National Tsing Hua University
Hsinchu, Taiwan
Republic of China

January 1977

U. S. ARMY RESEARCH AND DEVELOPMENT GROUP
FAR EAST
APO San Francisco 96343

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ABSTRACT

Chemical Modification of Cobrotoxin with Bifunctional Reagent 4,4'-Difluoro-3,3'-dinitro-diphenylsulfone

The two-dimensional structure of cobrotoxin has been established which permits a study of structure-function relationships. Previous studies on the chemical modification of cobrotoxin suggested that either the intact disulfide bonds or Tyr-25 are structurally important for maintenance of the active conformation of the toxin and at least two cationic groups held at certain distances in the molecule are functionally important for its neuromuscular blocking activity.

During the last decade, bifunctional reagents have been used for the study of protein structure in solution and also for the measurement of the distance between the sites which are cross-linked. The bifunctional reagent, 4,4'-difluoro-3,3'-dinitro-diphenylsulfone (FNPS), has been introduced into various biologically active proteins. It reacts preferentially with ε-NH_2 of lysine and phenolic OH group of Tyr-residues in the high pH reaction medium (pH 9.4 to 10.7).

The reaction of cobrotoxin with 4,4'-difluoro-3,3'-dinitrodiphenylsulfone (FNPS) was studied in an attempt to introduce new tertiary bonds into toxin. At pH 10.5, cobrotoxin was reacted with a 10-fold molar excess of FNPS at 25°C for 8 h. Dimers and higher molecular weight derivatives arising from intermolecular reactions were separated from intramolecularly cross-linked monomeric derivatives by gel filtration on a Sephadex G-50 column.

The monomeric fraction F III was further purified by chromatography on CM-cellulose column. Six peaks were obtained, of which three fractions, IIIa, IIIb and IIIc, were proved to be NPS-containing cobrotoxin derivatives and were all bifunctionally cross-linked with FNPS. The results of amino acid analyses of these derivatives revealed that the modification resulted in the cross-linkage between phenolic OH group of Tyr-residue and N-terminal α-NH_2 group (IIIa), and between Tyr-residue and ε-NH_2 group of Lys-residue (IIIb and IIIc). The lethality of IIIa, IIIb and IIIc decreased to 4 %, 4 % and 13 % with a concomitant increase in antigenic activities to 106.1 %, 123.9 % and 131.3 %, respectively.

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List of Appendixes

Chemical Modification of Cobrotoxin with Bifunctional Reagent 4,4'-Difluoro-3,3'-dinitro-diphenylsulfone

I. Introduction

The two-dimensional structure of cobrotoxin, crystalline neurotoxic protein isolated from the venom of Taiwan cobra Naja naja atra (Yang, 1965), has been established (Yang et al., 1969, 1970) which permits a study of structure-function relationships. Previous studies on the chemical modification of cobrotoxin suggested that either the intact disulfide bonds or Tyr-25 (Yang, 1967; Chang et al., 1971a) are structurally important for maintenance of the active conformation of the toxin and at least two cationic groups held at certain distances in the molecule are functionally important for its neuromuscular blocking activity (Chang et al., 1971b; Yang et al., 1974; Yang and Chang, 1976).

During the last twenty years, bifunctional reagents have been used for the study of protein structure in solution and also for the measurement of the distance between the sites which are cross-linked (Wold, 1971, 1972). The extinction coefficient of the resulted derivatives, either intramolecularly or intermolecularly cross-linked, in maximum absorption was also well defined (Tawde et al., 1963; Marfey et al., 1965; Gill et al., 1968).

The bifunctional reagent, 4,4'-difluoro-3,3'-dinitro-diphenylsulfone (FNPS), has been introduced into various biologically active proteins such as bovine serum albumin (Tawde et al., 1963; Wold, 1961a, 1961b), lysozyme (Stevens and Long, 1969), human CO hemoglobin (Macleod and Hill, 1970), and staphylococcal nuclease (Cuatrecasas et al., 1969). In these modification processes, FNPS reacts preferentially with ε-NH_2 of lysine and phenolic OH group of Tyr-residues in the high pH reaction medium (pH 9.4 to 10.7).

In this study, the raction of cobrotoxin with FNPS was carried out in an attempt to introduce new tertiary bonds

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Abbreviations: FNPS, 4,4'-difluoro-3,3'-dinitro-diphenylsulfone; NPS-, 3,3'-dinitro-diphenylsulfonyl; SDS, sodium dodecyl sulfate.

into the toxin. The modification resulted in the cross-linkage between phenolic OH group of Tyr-residue and the free N-terminal α-NH_2 group or ε-NH_2 group of Lys-residue. Intramolecularly cross-linked monomeric derivatives were separated from dimers and higher molecular weight polymers arising from intermolecular reactions by gel filtration on Sephadex G-50 column. The monomeric forms were further purified by chromatography on CM-cellulose column and the physicochemical properties as well as the biological activities of the NPS-cobrotoxin derivatives were studied.

II. Materials and Methods

Cobrotoxin used in this study was prepared from Taiwan cobra (Naja naja atra) venom as previously described (Yang, 1965). 4,4'-Difluoro-3,3'-dinitro-diphenylsulfone (FNPS), sodium dodecyl sulfate (SDS), N,N'-methylenebisacrylamide and constant boiling hydrochloric acid were purchased from Pierce Chemical Company. β-Alanine, glycine, Bromphenol blue, acrylamide and N,N,N',N'-tetramethylethylenediamine were obtained from Canal Industrial Corp., Coomassie brilliant blue R from Tokyo Chemical Industry Co., and Amidoblack 10 B from Fluka Ag, Co., Switzerland. Trypsin and chymotrypsin were the products of Worthington Biochemical Corp. Reagent grade β-mercaptoethanol and iodoacetic acid obtained from Matheson and Coleman Co. were used. Bovine serum albumin, ovalbumin, myoglobin, CM-cellulose and Sephadex G-25, G-50 were purchased from Sigma Chemical Co. and urea was a Mallinckrodt reagent. All other reagents were of analytical grade.

1. Reaction of Cobrotoxin with FNPS

35 mg of cobrotoxin (5 μmole) was dissolved in 5 ml of 1 % Na_2CO_3 solution (pH 10.5). A 10-fold molar excess of FNPS in 0.4 ml acetone was added dropwise at 25°C over a 30 min period with continuous stirring by using a magnetic stirrer. The reaction was allowed to proceed at 25°C for 8 h, then lyophilized.

2. Separation and Purification of NPS-cobrotoxin

The freeze-dried mixture was gel filtered on a 2.2 x

125 cm column of Sephadex G-50 equilibrated and eluted with 0.01 M ammonium acetate, pH 5.8. Each 5 ml fraction was collected at a rate of 20 ml per h. Optical density at 280 nm was measured by using a Beckman Model 25 spectrophotometer. The fractions of each peak were pooled and lyophilized. The monomeric fraction, F III, was further purified by CM-cellulose chromatography. The lyophilized material was dissolved in 2 ml of 0.01 M ammonium acetate buffer, pH 5.8 and applied onto a 2 x 26 cm column of CM-cellulose equilibrated with the same buffer. After complete adsorption, elution was conducted with a gradient of increasing salt concentration and pH from 0.01 M ammonium acetate, pH 5.8 to 0.5 M ammonium acetate, pH 6.8. Each 5 ml fraction was collected at a rate of 25 ml per h. Absorbance at 280 nm was recorded automatically by ISCO Model UA-5 absorbance monitor. Six peaks were obtained and the fractions of each peak were pooled and lyophilized.

3. Disc Electrophoresis

Disc electrophoresis on polyacrylamide gels was performed according to the method of Reisfeld et al. (1962).

4. SDS-gel Electrophoresis

SDS-gel electrophoresis for the determination of molecular weight by using 10 per cent polyacrylamide gels was performed essentially according to the method of Weber and Osborn (1969). The mobilities were plotted against the molecular weights of some standard proteins (bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsin, 24,000; myoglobin, 17,200 and cobrotoxin, 6,946) on a semi-logarithmic scale and the molecular weight of unknown substances could be estimated by interpolation.

5. Amino Acid Analysis

About 50 nmole of protein samples were hydrolyzed with constant boiling HCl (5.7 M) at 110°C for 24 h in evacuated sealed tubes. Amino acids were determined on a JEOL JLC-6AH fully automatic amino acid analyzer according to the procedure of Spackman et al. (1958).

6. Measurements of Lethal Activity

Lethality was measured by intraperitoneal injection of a serial 2-fold dilution of toxin into mice (16-18 g) with the amount of 0.2 ml per mouse as previously described (Yang, 1964). Six mice of both sexes were used for each dilution, and the $L\omega_{50}$ was calculated.

7. Immunological Procedures

Double diffusion in agar gel was performed by Ouchterlony's technique as previously described (Chang and Yang, 1969). The quantitative precipitating reactions were carried out as described by Kabat and Mayer (1961). Increasing amounts of toxin (5-45 μg) in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl were added to a constant amount (0.3 ml) of antisera in total volume of 1 ml. The tubes were incubated for 30 min at 37°C and then left overnight at 4°C. The precipitates were washed 3 times with cold 0.15 M NaCl, after which they were dissolved in 3 ml of 0.1 M NaOH, and the absorbances were measured at 280 nm.

III. Results

1. Chemical modification of cobrotoxin with FNPS

Cobrotoxin 35 mg (5 μmole) in 5 ml of 1 % Na_2CO_3 solution (pH 10.5) was reacted with a 10-fold molar excess of FNPS at 25°C for 8 h, then gel filtered on a column of Sephadex G-50. The first three fractions shown in Fig. 1 contained protein and the materials eluting after 360 ml (F IV) consisted of low molecular weight reagents and buffer constituents.

In a control experiment, cobrotoxin was exposed to the same buffer at pH 10.5 for 8 h without FNPS. The subsequent gel filtration on Sephadex G-50 gave a single peak corresponds to that of the native cobrotoxin (Fig. 1), indicating that no aggregation had occurred. F III eluted at the same effluent volume as those of native cobrotoxin and control experiment is, therefore, monomeric forms which account for 60.1 per cent of the protein. F I and F II are the dimers and polymerized derivatives arising from

the intermolecular reactions between cobrotoxin and FNPS.

As seen from Fig. 2, the SDS-gel electrophoresis patterns show clearly that the main components of F III are monomeric forms, although a trace amount of dimeric derivatives exist. On the other hand, the main components of F II are dimers with a small amount of monomeric derivatives and some higher molecular weight polymers. Similarly, F I consists of polymeric derivatives with the major molecular weight populations of 23,000, 29,000 and 36,000 with a small amount of other higher molecular weight polymers and a trace amount of dimers, as estimated by interpolation on electrophoretic mobility of marker proteins (Fig. 3).

2. CM-cellulose chromatography of NPS-cobrotoxin

The combined eluates of the F III obtained from duplicate Sephadex G-50 (cobrotoxin 70 mg, 10 μmole) chromatography were freeze-dried and applied on a 2 x 26 cm column of CM-cellulose. As shown in Fig. 4-B, six peaks, IIIa, IIIb, IIIc, IIId, IIIe and IIIf, were obtained. Under the same condition, control experiment on cobrotoxin gave a main peak corresponding to the position of native cobrotoxin and two additional peaks in the anodic side (Fig. 4-A). These three peaks just correspond with the peaks, IIId, IIIe and IIIf, on the CM-cellulose chromatogram of F III (Fig. 4-B). Therefore, IIId, IIIe and IIIf are the non-reacted toxin fractions and IIIa, IIIb and IIIc might be the NPS-containing toxin derivatives.

It is our desire to get higher yield of monomeric forms of NPS-toxin derivatives and less polymerized forms and non-reacted toxin fractions. For this reasons, several conditions were tried. The CM-cellulose chromatographic profiles of the monomeric forms derived from three different experimental conditions are shown in Fig. 4 and the yields of each fraction are presented in Table I. It is obvious that the most favourable condition to get higher yield of the monomeric forms of NPS-cobrotoxin derivatives is to react cobrotoxin with FNPS in 5 ml of 1 % Na_2CO_3 buffer (pH 10.5) at 25°C for 8 h.

3. Characterization of the FNPS modified cobrotoxin derivatives

As seen from Fig. 2, although the F III showed a faint band at the position corresponds to dimeric derivatives besides the main band of monomeric forms, the six fractions (IIIa, IIIb, IIIc, IIId, IIIe and IIIf) obtained from CM-cellulose chromatography all revealed as a single band on the same position as native cobrotoxin on SDS-polyacrylamide gels. This indicates that IIIa, IIIb and IIIc are intramolecularly cross-linked monomeric derivatives.

As shown in Fig. 5, fractions IIIa through IIIf all revealed as a single band on polyacrylamide gels at pH 4.5. The position of each fraction appeared on the gel was also well correspondent to the chromatographic profile of CM-cellulose column. In addition, the combined mixture of IIIf and native cobrotoxin gave a single band on the gel, show its identity to native cobrotoxin.

It is very interesting to note that although the lethality of the NPS-cobrotoxin derivatives, IIIa, IIIb and IIIc, retained only 4 %, 4 % and 13 %, respectively, their antigenic activity increased to 106.1 %, 123.9 % and 131.3 %, respectively (Table II, Fig. 6). However, IIIf retained essentially full lethality and antigenic activity. IIId and IIIe showed somewhat decrease in lethality and antigenic activity. F I from Sephadex G-50, the higher molecular weight polymers, gave none of precipitates in immune precipitin reaction and lost almost completely the lethality, however, F II gave even a little increase in precipitin reaction and retained about 4 % of lethal activity.

4. Amino acid composition of FNPS modified cobrotoxin derivatives

The amino acid composition of the FNPS modified toxin derivatives are compared with that of cobrotoxin (Table III) and the results are as follows:

IIIa: 0.81 mole of leucine and 0.97 mole of Tyr-residue are modified, suggesting that there is a covalent cross-linkage between a phenolic OH group of tyrosine and the α-NH_2 group of N-terminal leucine.

IIIb: One mole each of Lys- and Tyr-residues are modified, suggesting that there is a covalent cross-linkage between tyrosine and ε-NH_2 group of lysine.

IIIc: 1.15 moles of lysine and 0.85 mole of Tyr-residues are modified, suggesting that there are possibilities of covalent cross-linkage between lysine and tyrosine and also, in part, between two ε-NH_2 groups of Lys-residues in a probable ratio of 0.85 to 0.15.

IIId and IIIe: There are a significant decrease in aspartic content (2.5 residues in IIId and 2 in IIIe), suggesting that a possible destruction of Asn-residues in cobrotoxin (6 Asn-residues) may occur at high pH reaction medium.

IIIf: The amino acid composition of IIIf is essentially identical to that of native cobrotoxin, supporting the evidence of IIIf is the unreacted cobrotoxin.

IV. Discussion

The results of SDS-gel electrophoresis revealed that the reaction products of bifunctional chemical modification are very complex. The complexity was found to be dependent on the molar ratio of the reactants, protein and FNPS, volume of reaction mixture, and the reaction time and temperature. The reaction of human CO hemoglobin with FNPS was recommended to carry out in 300 ml of reaction mixture (Macleod and Hill, 1970), the reaction of bovine pancreatic ribonuclease A with 1,5-difluoro-2,4-dinitrobenzene in 1.8 l of $NaHCO_3$ (Marfey et al., 1965) and the reaction between FNPS and lysozyme was proceeded in 3.5 l of 1 % Na_2CO_3 (Stevens and Long, 1969). The experimental condition used in this study was chosen in such a manner to get a higher yield of intramolecularly cross-linked monomeric derivatives. When compared the several different conditions tried for the reaction of cobrotoxin with FNPS (Table I), the best condition was found to be in a small volume, 5 ml, of 1 % Na_2CO_3 at 25°C for 8 h.

The polymers produced in the reaction of bovine

pancreatic ribonuclease A with 1,5-difluoro-2,4-dinitrobenzene are believed to be noncovalent aggregates generated by the lyophilization (Marfey et al., 1965). However, no aggregates were formed in the reaction of cobrotoxin with tetranitromethane and trinitrobenzene sulfonate (Chang et al., 1971a, 1971b) and also during the control experiment of cobrotoxin in this study. These facts suggest that the formation of dimers and higher molecular weight polymers during the reaction between cobrotoxin and FNPS is due to the intermolecular cross-linkage by FNPS.

The spectra of IIIa, IIIb and IIIc in the range of 360 to 420 nm are the same in the extreme acid (pH 1.5) and alkaline medium (pH 11.5) (Fig. 7) and the spectra remain unchanged upon standing in the alkaline medium at pH 11.5 for 10 h. This indicates that both fluorine atoms of FNPS are substituted bifunctionally in the above three cross-linked derivatives (Marfey et al., 1965). The other three fractions, IIId, IIIe and IIIf, appear to be non-reacted toxins. Their spectra in the region between 260 and 500 nm are almost identical to that of the native cobrotoxin.

The biological activities of the six fractions from CM-cellulose column are different. The non-reacted fraction, IIIf is almost fully active as native cobrotoxin. However, IIId and IIIe showed somewhat decrease in their biological activities (Table II). The pronounced decrease of lethality of the NPS-cobrotoxin derivatives, IIIa, IIIb and IIIc, and their concomitant increase in antigenic activity may be attributable to a direct interference with the toxic site(s) incurred by the introduction of the NPS-group(s) and to a conformational change occurred to the molecule.

Bifunctional reagent, FNPS, reacts preferentially with amino groups and tyrosine phenolic OH groups, but sulfhydryl and imidazole groups can also react (Wold, 1972). However, the pKa values of these groups are 10.53 (ε-NH_2 of lysine), 10.07, 8.5, and 6.0, respectively. Therefore, the reaction between protein and FNPS is favored to the amino and phenolic OH groups under high pH (above 10) reaction medium (Tawde et al., 1963; Wold, 1961a, 1961b; Stevens and Long, 1969; Macleod and Hill, 1970; Cuatrecasas et al., 1969). The results of amino acid analyses of the

Tyr-35, and in a possible ratio of 0.15 to 0.85.

In order to identify the amino acid residues reacted with FNPS, the modified derivatives were digested with trypsin after reduction and S-carboxymethylation. The digests were separated by a combination of high-voltage paper electrophoresis and descending paper chromatography. The NPS-peptides on the peptide map were eluted for amino acid analysis and the work is now in progress.

V. Conclusion

The reaction of cobrotoxin with 4,4'-difluoro-3,3'-dinitrodiphenylsulfone (FNPS) was studied in an attempt to introduce new tertiary bonds into toxin. At pH 10.5, cobrotoxin was reacted with a 10-fold molar excess of FNPS at 25°C for 8 h. Dimers and higher molecular weight derivatives arising from intermolecular reactions were separated from intramolecularly cross-linked monomeric derivatives by gel filtration on a Sephadex G-50 column.

The monomeric fraction F III was further purified by chromatography on CM-cellulose column. Six peaks were obtained, of which three fractions, IIIa, IIIb and IIIc, were proved to be NPS-containing cobrotoxin derivatives and were all bifunctionally cross-linked with FNPS. The results of amino acid analyses of these derivatives revealed that the modification resulted in the cross-linkage between phenolic OH group of Tyr-residue and N-terminal α-NH_2 group (IIIa), and between Tyr-residue and ε-NH_2 group of Lys-residue (IIIb and IIIc). The lethality of IIIa, IIIb and IIIc decreased to 4 %, 4 % and 13 % with a concomitant increase in antigenic activities to 106.1 %, 123.9 % and 131.3 %, respectively.

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APPENDIX A-1

Table I

The Yields of FNPS Modified Toxin Derivatives in Three Different Conditions

Experimental condition		in 5 ml at 25°C for 8 h Yield (%)	in 5 ml at 4°C for 8 h Yield (%)	in 350 ml at 24°C for 24 h Yield (%)
Gel Filtration on Sephadex G-50	F I	21.3	35.3	10.0
	F II	10.3	16.8	22.3
	F III	60.1	40.8	67.9
Chromatography of F III on CM-cellulose	IIIa	5.7	0.3	4.4
	IIIb	4.2	0.1	-
	IIIc	9.1	0.3	6.1
	IIId	13.4	13.0	-
	IIIe	3.5	3.2	38.0
	IIIf	2.2	16.6	19.4

APPENDIX A-2

Table II

Lethality and Antigenic Activity of Monomer Fractions Obtained from CM-cellulose Chromatography

Fractions	Lethality (%)	Antigenic activity (%)
IIIa	4	106.1
IIIb	4	123.9
IIIc	13	131.3
IIId	87	65.5
IIIe	87	73.3
IIIf	96	96.1

APPENDIX A-3

Table III

Amino Acid Composition of Cobrotoxin and NPS-cobrotoxin Derivatives

Amino Acid	Residues per mole of protein						
	Cobrotoxin	NPS-cobrotoxin					
		IIIa	IIIb	IIIc	IIId	IIId	IIIe
Aspartic acid	8	7.81	7.93	7.92	5.50	6.00	8.02
Threonine	8	7.91	7.91	7.94	8.08	8.02	8.01
Serine	4	4.03	4.08	4.11	4.05	4.03	4.01
Glutamic acid	7	7.14	7.09	7.10	6.94	7.05	7.03
Proline	2	2.21	2.05	2.09	2.04	1.89	1.92
Glycine	7	7.08	7.05	7.03	7.03	7.09	7.02
Alanine	-	-	-	-	-	-	-
Half-cystine	8	8.10	7.93	7.95	8.10	8.04	8.10
Valine	1	1.03	1.02	1.20	0.98	1.23	1.02
Methionine	-	-	-	-	-	-	-
Isoleucine	2	2.00*	2.00	2.00	2.00	2.00	2.00
Leucine	1	0.19	1.02	1.00	1.04	1.00	1.07
Tyrosine	2	1.03	1.01	1.15	2.05	2.10	1.94
Phenylalanine	-	-	-	-	-	-	-
Lysine	3	2.95	2.01	1.83	3.03	3.02	3.03
Histidine	2	1.98	1.98	2.01	2.00	1.92	1.99
Arginine	6	6.02	5.91	5.98	5.97	5.98	6.03

* All values are expressed as molar ratios based on isoleucine = 2

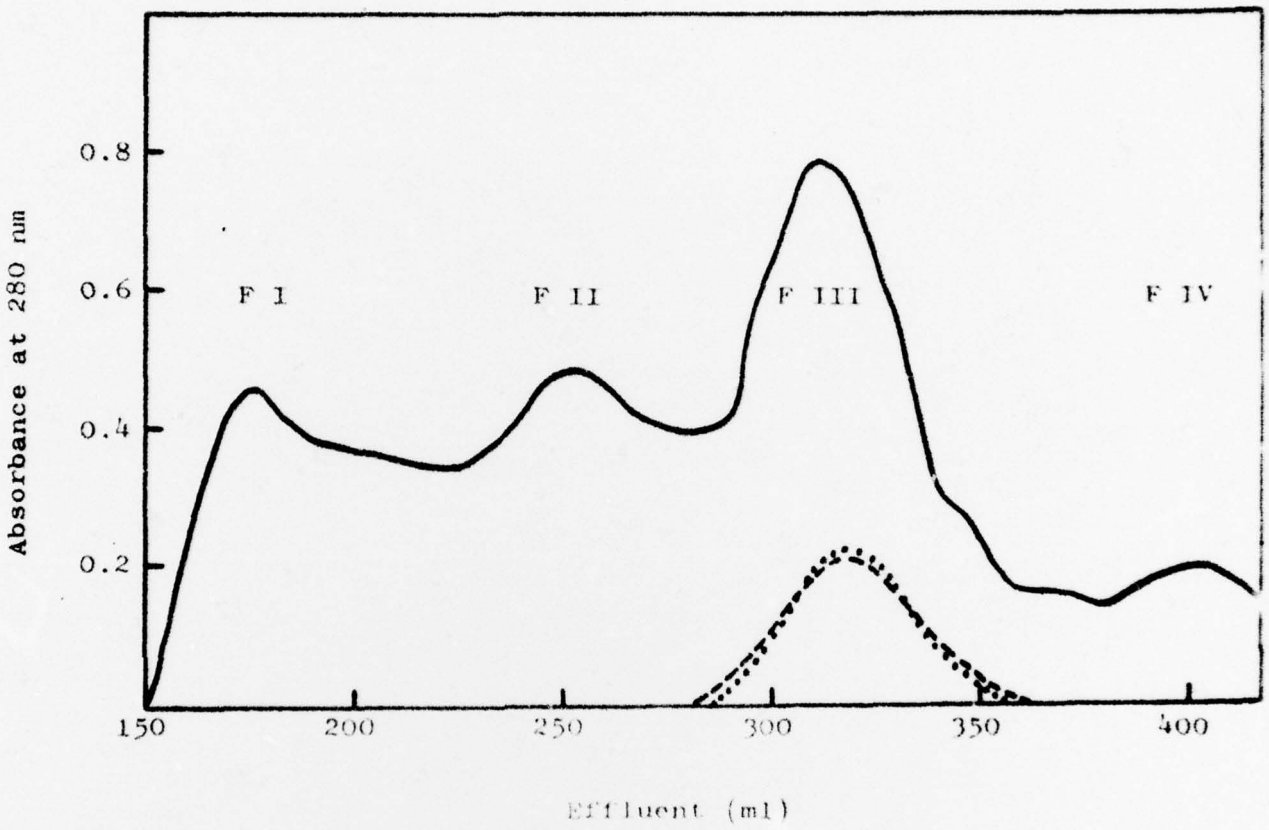

Fig. 1. Gel filtration of the FNPS reacted cobrotoxin on Sephadex G-50.

The column was equilibrated with 0.01 M ammonium acetate buffer, pH 5.8, to a constant height (2.2 x 125 cm). Sample was dissolved in 2 ml of the same buffer and applied on the column and eluted with the same buffer. The flow rate was 20 ml per h and fractions of 5 ml were collected. ———, FNPS reacted cobrotoxin; -----, control experiment; ·····, native cobrotoxin.

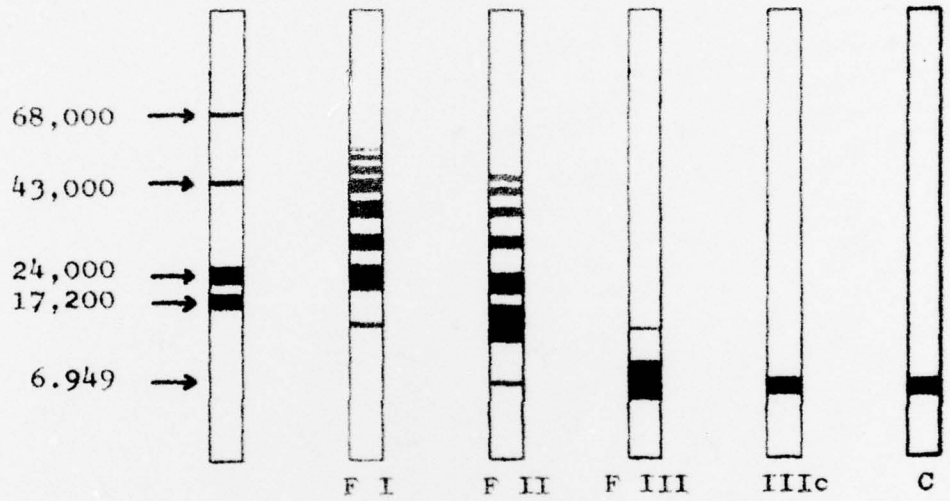

Fig. 2. SDS-gel electrophoresis of FNPS modified toxin derivatives.

The SDS-gel electrophoresis was performed in 10 % polyacrylamide gels. Electrophoresis was run at 8 mA per gel for 4 h. The amount of protein applied to each gel was 25, 30, 10, 10 and 10 µg, respectively, for F I, F II, F III, IIIc and cobrotoxin.

APPENDIX B-3

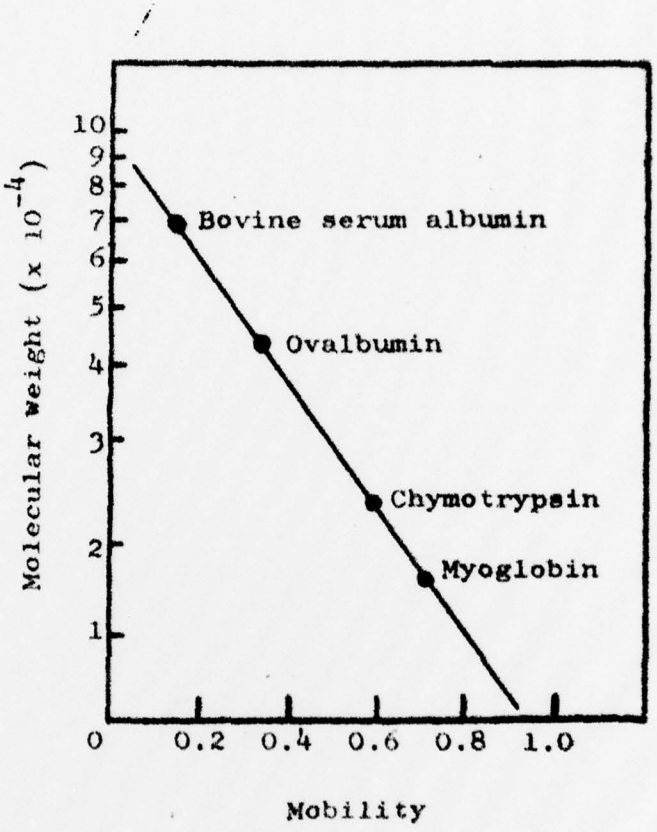

Fig. 3. Electrophoretic mobility of marker proteins.

Determination of the molecular weight of FNPS-modified toxins by SDS-gel electrophoresis was performed in 10 % polyacrylamide gels as described in Fig. 2. Mobilities were calculated as follows:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}} \times \frac{\text{length before staining}}{\text{length after destaining}}$$

The mobilities were plotted against the molecular weights of the standard proteins (bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsin, 24,000; myoglobin, 17,200; cobrotoxin, 6,949) on a semi-logarithmic scale and the molecular weight of the FNPS-modified toxins were estimated by interpolation.

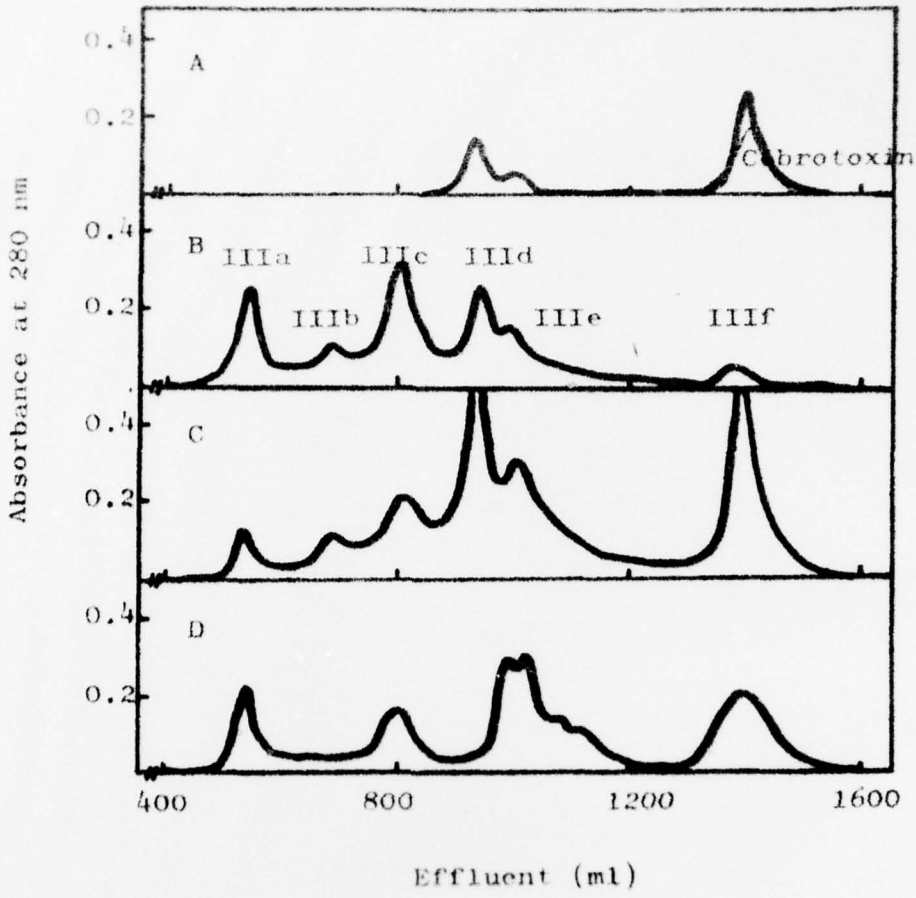

Fig. 4. CM-cellulose column chromatography of F III from Sephadex G-50 run shown in Fig. 1.

A: A control experiment of cobrotoxin (20 mg) from Sephadex G-50 was dissolved in 1 ml of 0.01 M ammonium acetate, pH 5.8, and applied to a 2 x 26 cm column of CM-cellulose equilibrated with the same buffer. Elution was conducted with a gradient of increasing salt concentration and pH from 0.01 M ammonium acetate, pH 5.8, to 0.5 M ammonium acetate, pH 6.8. Each 5 ml fraction was collected at a rate of 20 ml per h.

B: Combined elutes of F III from duplicate runs on Sephadex G-50 were chromatographed on CM-cellulose column. The reaction condition used for cobrotoxin and FNPS was in 5 ml of 1 % Na_2CO_3 buffer, pH 10.5, at 25°C for 8 h.

C: The reaction condition was in 5 ml of 1 % Na_2CO_3 buffer, pH 10.5, at 4°C for 24 h.

D: The reaction condition was in 350 ml of 1 % Na_2CO_3 buffer, pH 10.5, at 4°C for 24 h.

APPENDIX B-5

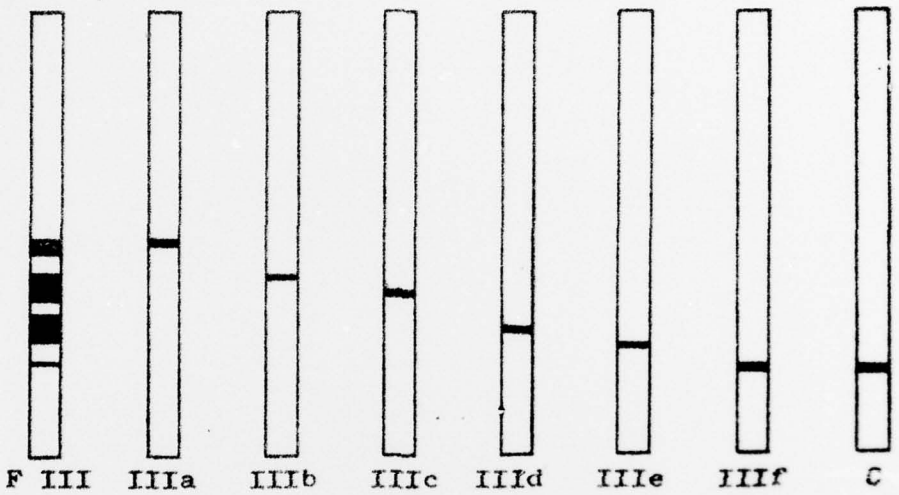

Fig. 5. Disc electrophoresis of F III and six fractions from CM-cellulose column shown in Fig. 4-B.

Disc electrophoresis in 7 % polyacrylamide gel was carried out in small glass tubes, 5 x 75 mm. After the preruns by applying a current of 1.5 mA per gel for 30 min, the protein sample (10 μg) mixing with a trace of tracking dye was applied and stacking ran 2 mA per gel for 30 min. The electrophoresis was performed at 15°C by applying a current of 8 mA per gel for 45 min until the dye moved to the buttom of the gel tubes.

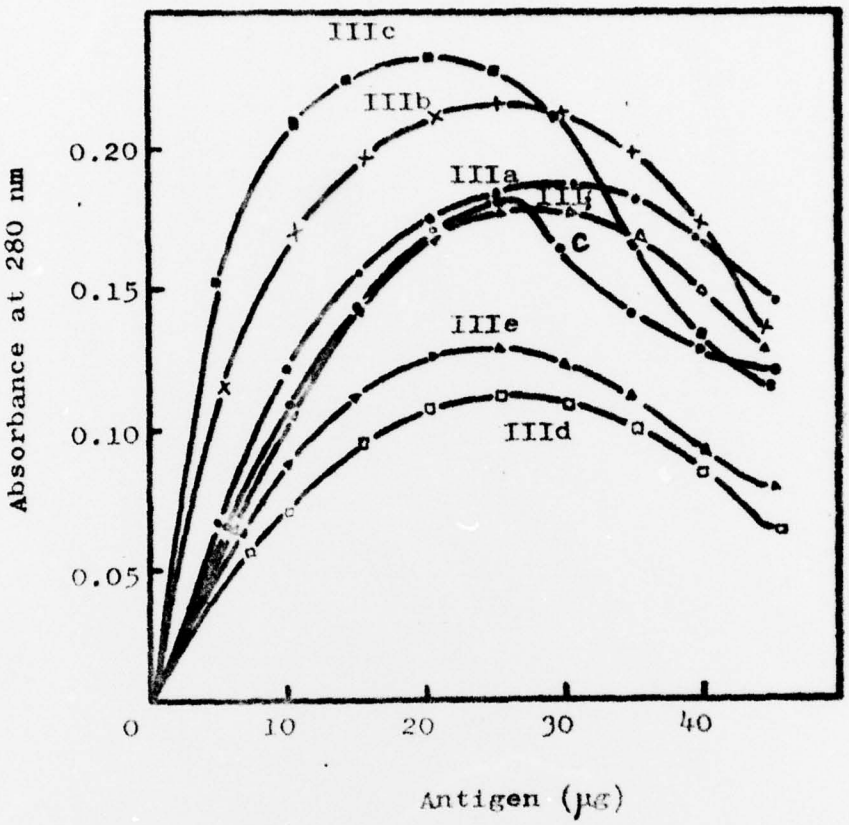

Fig. 6. Quantitative precipitin reactions of FNPS modified derivatives with anti-cobrotoxin serum as compared with cobrotoxin. 0.3 ml of antiserum was used in each case. ○——○, cobrotoxin; ●——●, IIIa; ×——×, IIIb; ■——■, IIIc; ■——□, IIId; ▲——▲, IIIe; △——△, IIIf.

APPENDIX B-7

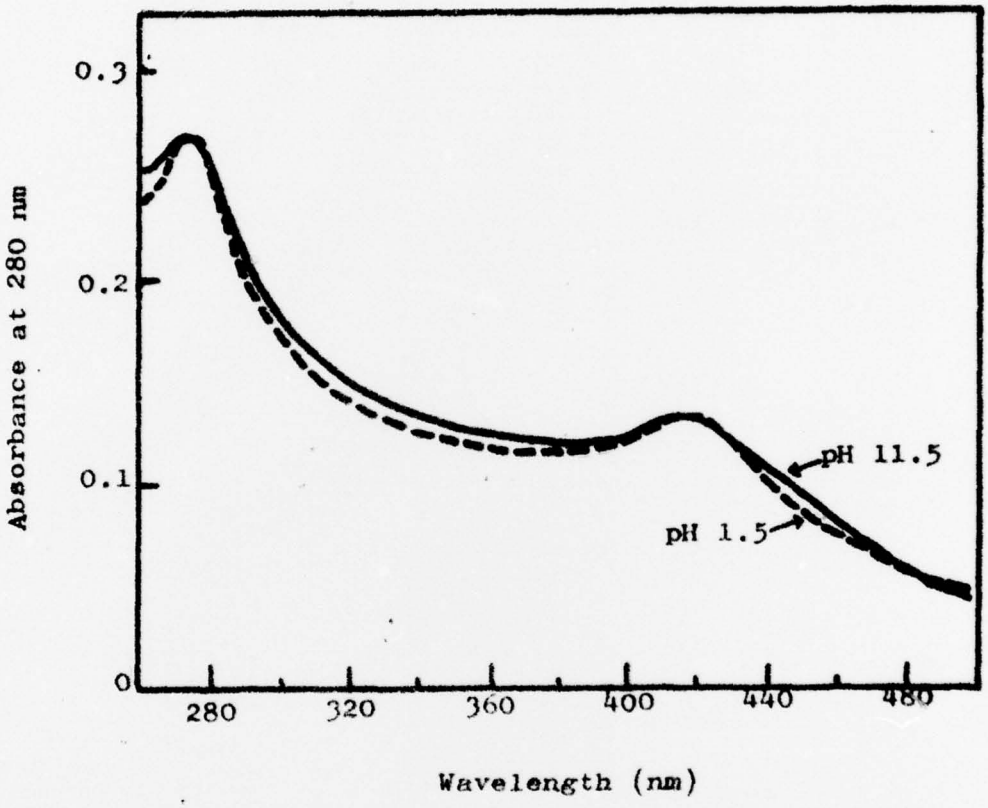

Fig. 7. Absorption spectra of IIIc from CM-cellulose column chromatography at pH 1.5 and pH 11.5.

Protein concentration was about 0.12 mg per ml.

APPENDIX B-8

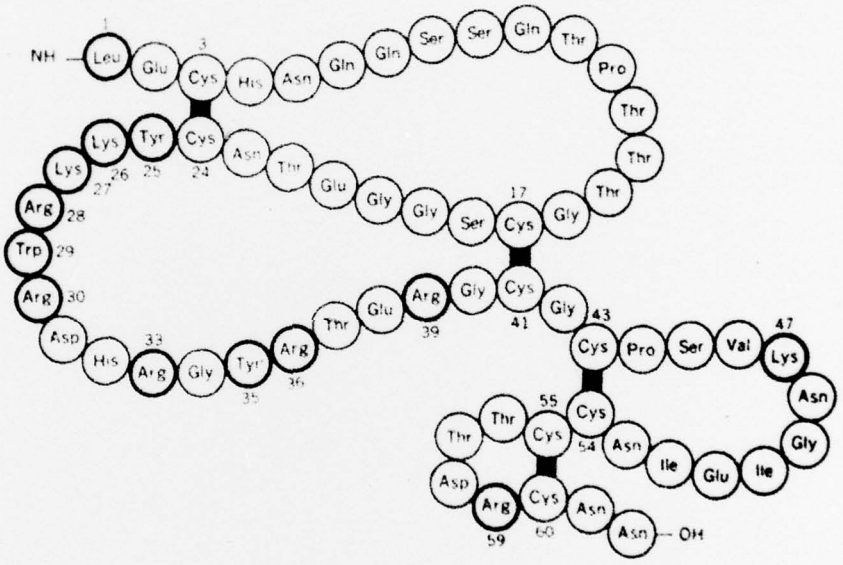

Fig. 8. Structure of cobrotoxin.

Two-dimensional schematic diagram showing the arrangement of the disulfide bonds and the sequence of the amino acid residues.

List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to lethality. Biochim. Biophys. Acta 133 (1967) 346-355.

2. Optical rotatory dispersion of cobrotoxin. J. Biochem. 61 (1967) 272-274.

3. Biochemical studies on the toxic nature of snake venom. Intern. Congr. Biochem. 7th, Tokyo Col. VIII, 1 (1967).

4. Studies on fluorescent cobrotoxin. Biochim. Biophys. Acta 147 (1967) 600-602.

5. Study on ^{131}I labeled cobrotoxin. Toxicon 5 (1968) 295-301.

6. Optical rotatory dispersion and circular dichroism of cobrotoxin. Biochim. Biophys. Acta 168 (1968) 373-376.

7. Amino acid composition and end group analysis of cobrotoxin. Toxicon 7 (1969) 43-47.

8. Immunochemical studies on cobrotoxin. J. Immun. 102 (1969) 1437-1444.

9. The amino acid sequence of cobrotoxin. Biochim. Biophys. Acta 188 (1969) 65-77.

10. Biochemical and immunochemical studies on cobrotoxin. The Snake 2 (1970) 1-12.

11. Structure-activity relationships and immunochemical studies on cobrotoxin. Radiation Sensitivity of Toxins and Animal Poisons IAEA-PL-334/6 (1970) 63-74.

22. Chemical synthesis of a peptide with cobrotoxin activity. Animal, Plant, and Microbial Toxins (Ohsaka, A., Hayashi, K. and Sawai, Y., eds.) Plenum Pub. Corp., New York, Vol. 2 Chemistry, Pharmacology, and Immunology (1976) 89-92.

23. A fluorescent study of the neurotoxic effect of cobrotoxin on the cholinergic reaction of acetylcholine with synaptic membranes. Animal, Plant, and Microbial Toxins (Ohsaka, A., Hayashi, K. and Sawai, Y., eds.) Plenum Pub. Corp., New York, Vol. 2 Chemistry, Pharmacology, and Immunology (1976) 169-178.

24. Structure and function of cobrotoxin. The Snake 8 (1976) 57-63.

25. Purification of anticobrotoxin antibody by affinity chromatography. Toxicon in press.

26. The reaction of cobrotoxin with the bifunctional reagent 4,4'-difluoro-3,3'-dinitro-diphenylsulfone (FNPS). Proc. 5th Intern. Symp. on Animal, Plant, and Microbial Toxins (Rosenberg, P., ed.) Pergamon Press, Oxford, in press.

List of Graduate Students

Chun-chang Chang, D. Sc.

Lung-hsiung Hsu, Ph. D.

Che-an Yang, M. S.

Yea-shiang Su, B. S.

Hung-chih Chiu, B. S.

Ing-fong Liou, M. S.

Hsing-ming Chen, B. S.

Shu-Mei Yao, B. S.

Chia-po Lin, M. S.

Ming-fong Lin, M. S.

Hong-yu Lee, B. S.

Lee-chu Chang, B. S.

Shin-kuang Liu, B. S.